

PREDICTORS OF INVASION IN DUCTAL CARCINOMA IN SITU OF THE BREAST: THE VALUE OF A SCORING SYSTEM

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Ductal carcinoma in situ (DCIS) of the breast shows unpredictable association with invasive ductal carcinoma (IDC). Comedo DCIS (CDCIS) is more frequently associated with IDC than noncomedo DCIS (NCDCIS). We studied prognostic variables in 64 cases of DCIS to identify predictors of invasion. These factors included DCIS type and nuclear grade and two counts of the AgNOR silver staining technique for identification of ploidy and proliferative activity (PA) using two counts: mAgNOR for ploidy and pAgNOR for PA. The other factors included immunostaining of the lesions for epidermal growth factor receptors (EGFR), cathepsin-D (C-D), and the c-erbB-2 oncogene. The 34 cases associated with IDC had pAgNOR ranging from 3% to 36% (median 11%), whereas cases not associated with IDC had a pAgNOR range of 0% to 25% (median 5%; $P=0.0001$). The correlation between mAgNOR and the development of IDC was less statistically significant. The DCIS type and staining pattern for EGFR, C-D, and c-erbB-2 showed no statistical correlation of individual variables with the development of IDC. A scoring system adding the values of the seven variables was used. A score of 1-2 was given to each variable, depending on whether it was positive or negative. Lesions associated with IDC had a median total score of 8 (± 1.35 SD), whereas those lesions not associated with invasion had a median score of 4 (± 1.45 SD; $P=0.0002$). We conclude that proliferative activity analysis may play a significant role in predicting the invasive potential of DCIS. The use of a scoring system adding the sum of single prognostic indicators may provide more useful information regarding the prediction of invasive potential of DCIS than single indicators. *Ann Saudi Med* 1997;17(4):427-431.

Ductal carcinoma in situ (DCIS) represents approximately 70% of noninvasive breast tumors.¹ The lesions represent a high proportion of mammographically detected non-palpable breast lesions.² Although DCIS is not uniformly associated with invasive carcinoma of the breast, a significant proportion of these lesions progress to or coexist with invasive cancer.³⁻⁹ Lymph node metastasis can even be found in association with DCIS without any histological evidence of invasion.¹⁰ Several attempts have been made to identify prognostic indicators predicting the risk of invasion associated with DCIS. Histological classification of DCIS into comedo (CDCIS) and noncomedo (NCDCIS) subtypes is the most popular and technically feasible method.¹¹ Other prognostic indicators have been studied in invasive ductal carcinoma, and seem to have a significant impact on predicting aggressive behavior of invasive ductal carcinoma. These factors include proliferative activity analysis using the argyrophilic nucleolar organizer (AgNOR) silver stain,¹² oncogene expression, such as c-erbB-2,¹³ epidermal

growth factor receptors (EGFR),¹⁴ and the proteolytic enzyme cathepsin-D.

Because there is a need to identify indicators predicting invasion in DCIS, we studied the above parameters in cases of pure DCIS and cases of combined DCIS and invasive ductal carcinoma to see if we could identify the most significant single factor predicting invasion. We also used a scoring system combining all the factors to assess the predictive value of the combined factors.

Materials and Methods

Sixty-four cases of ductal carcinoma in situ of the breast were selected for this study. The cases were retrieved from the files of the University of Alberta Hospital ($n=37$), and the Case Western Reserve University hospitals ($n=27$). The cases were either of pure ductal carcinoma in situ or ductal carcinoma in situ associated with invasive ductal carcinoma. Classification of the ductal carcinoma in situ into comedo and noncomedo subtypes was based on well-established histological criteria.^{16,17} Grading of the nuclei in the carcinoma in situ into high and low grades was also done based on criteria in the literature.^{18,19} Sections of formalin-fixed paraffin-embedded tissue were submitted for the AgNOR silver stain and immunohistochemistry for cathepsin-D, the c-erbB-2 oncogene and epidermal growth

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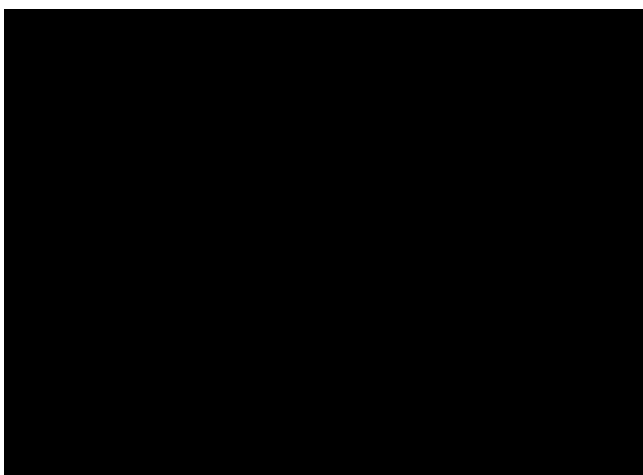


FIGURE 1. A case of ductal carcinoma in situ showing positive cytoplasmic membrane staining for epidermal growth factor receptor (Immunoperoxidase, 25x).

factor receptors (EGFR).

The AgNOR silver staining technique was performed using the modified technique of Ploton,²⁰ further modified by Crocker.²¹ Two AgNOR granule counts were performed. The first count consisted of the mean number of AgNOR granules in 100 cells counted (mAgNOR), and the second count was the percentage of nuclei exhibiting ≥ 5 AgNOR granules/nucleus. The first count corresponded to ploidy and the second count corresponded to proliferative activity.²²⁻²⁵ The counts were performed in the areas of ductal carcinoma in situ, with a minimum of four fields chosen for count in each lesion. Any AgNOR clumps not discernible at light microscopy were considered one single granule. This method of count is referred to as method B by Suresh et al.²⁶ This is in contrast to method A, where an attempt is made to count all AgNOR granules. The count in method B is usually lower than in method A.

The immunoperoxidase technique was used for the identification of cathepsin-D, EGFR and the c-erbB-2 oncogene product. Monoclonal antibodies were used (Vector Laboratories, Burlingame, California, U.S.A). Any positive staining for any of the antibodies was considered positive for expression of the corresponding antigen.

A scoring system that added seven variables to obtain the total score for each case was used. The variables included the presence or absence of comedo carcinoma, nuclear grade, aneuploid mAgNOR counts (≥ 2.4), pAgNOR $\geq 8\%$, EGFR, cathepsin-D, and c-erbB-2. When the parameter was negative, a score of 1 was given and score of two was given to each positive parameter. The total score ranged from 7-14.

Statistical analysis was performed using the Kruskal-Wallis analysis of variance.²⁷ A statistical software (*True Epistat*TM, Richardson, Texas, USA) and a 486 personal

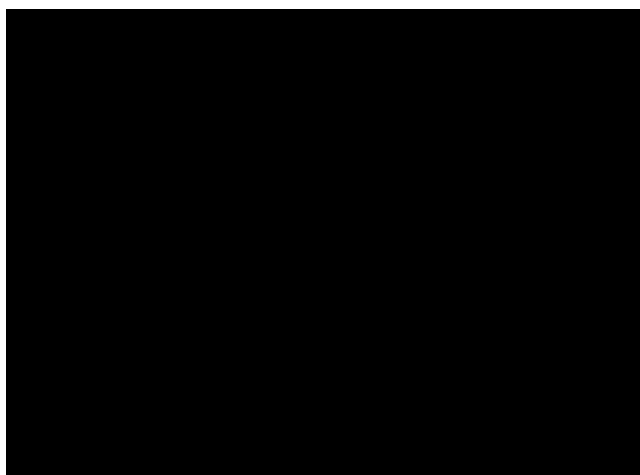


FIGURE 2. A case of ductal carcinoma in situ of the breast showing positive immunolabeling for c-erbB-2 highlighting the cytoplasmic membrane similar to epidermal growth factor receptor (Immunoperoxidase, 25x).

computer were used for the statistical analysis. The analysis was performed to identify the statistical difference between each individual parameter in pure ductal carcinoma in situ, and that not associated with invasion. The analysis was also used to identify the difference between the total score in DCIS not associated with invasion and those cases associated with invasion.

Results

The patient ages ranged from 38 to 77 years, with a median age of 48 years. The 64 cases included 30 cases of pure ductal carcinoma in situ, and 34 cases of ductal carcinoma in situ associated with invasion. Patients with invasive carcinoma had either synchronous or metachronous development of invasive ductal carcinoma. Patients with pure ductal carcinoma in situ were followed for a period ranging from 13 to 36 months (median 18 months) for the possible development of invasive carcinoma.

Immunohistochemical staining for EGFR showed a cytoplasmic membrane staining pattern (Figure 1). Seven of the 34 cases of DCIS associated with invasion showed EGFR positivity (20%), whereas only four of the 30 cases not associated with invasion showed positive staining (13%). No statistical difference was seen between the two groups. C-erbB-2 showed a staining pattern similar to that seen with EGFR (Figure 2). Thirteen of the 34 cases of DCIS associated with invasion exhibited positivity for c-erbB-2 (38%), whereas only six of the 30 cases not associated with invasion (20%) showed such a pattern. No statistically significant difference could be seen between the two groups of patients. Cathepsin-D positive staining was mainly seen as a granular cytoplasmic staining (Figure

3). Twenty cases of DCIS associated with invasion (58%) showed positive staining, whereas only 12 of 30 cases of DCIS not associated with invasion (35%) showed such expression. The difference was not statistically significant.

Nineteen of the 34 cases of DCIS associated with invasive carcinoma (55%) showed an mAgNOR pattern suggestive of aneuploidy (≥ 2.4), whereas aneuploidy was seen in only eight of the 30 cases of DCIS not associated with invasive carcinoma (26%). The difference was statistically significant ($P=0.01$). Cases of DCIS associated with invasion showed a pAgNOR ranging from 3% to 36%, with a median of 11% (Figure 4), whereas cases not associated with invasion had pAgNOR of 0 to 25% with a median of 5%. The difference was statistically significant ($P=0.001$). Cases exhibiting aneuploidy usually showed significant increase in pAgNOR counts and granule distribution (Figure 5).

When the total score combining all variables was added, cases of DCIS associated with invasion had a median score of 8, whereas those cases not associated with invasion had a median score of 4. The difference was statistically significant ($P=0.00002$).

Discussion

Cathepsin-D is a proteolytic enzyme belonging to the family known as aspartate proteinases.^{28,29} Expression of cathepsin-D has been variably correlated with poor prognosis in invasive breast carcinoma. Expression of the enzyme may suggest increased invasive potential. Although there was a higher number of cases where cathepsin-D was expressed when invasion was present, we did not find a significant correlation between cathepsin D expression as an isolated parameter and the potential for invasion. Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein whose expression is important in the regulation of breast cancer cell growth.³⁰ The activity of EGFR has been closely related to c-erbB-2 oncogene expression, as well as increased proliferation of tumor cells. These findings were mainly seen in invasive ductal carcinoma. C-erbB-2 oncogene activity has been linked to protein kinase activity.^{31,32} The gene product has structural homology with EGF and its receptor (EGFR). Studies have shown that this oncogene is amplified (overexpressed) in more aggressive forms of DCIS.¹³ We have also noticed that immunohistochemical expression of c-erbB-2 and EGFR are very similar, confirming the close relationship between the two proteins. However, we were not able to show any significant value of either EGFR or c-erbB-2 expression in predicting invasive potential of DCIS.

The classification of DCIS into comedo and non-comedo variants has shown a promise for identifying more aggressive variants of DCIS.¹⁸ The method is simple,

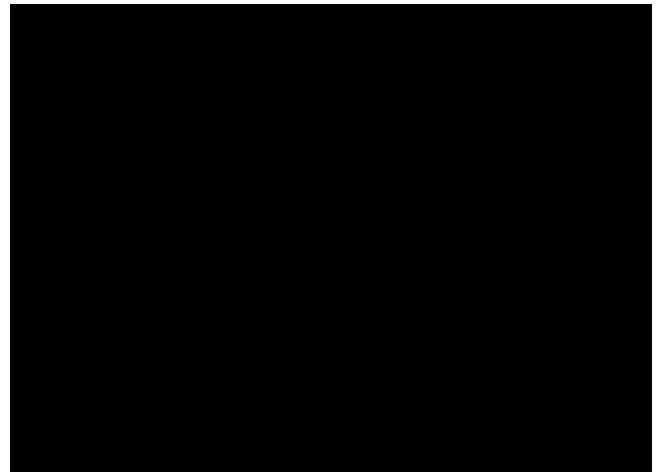


FIGURE 3. Case of ductal carcinoma in situ of the breast showing positive granular cytoplasmic staining for cathepsin-D (Immunoperoxidase, 25X).

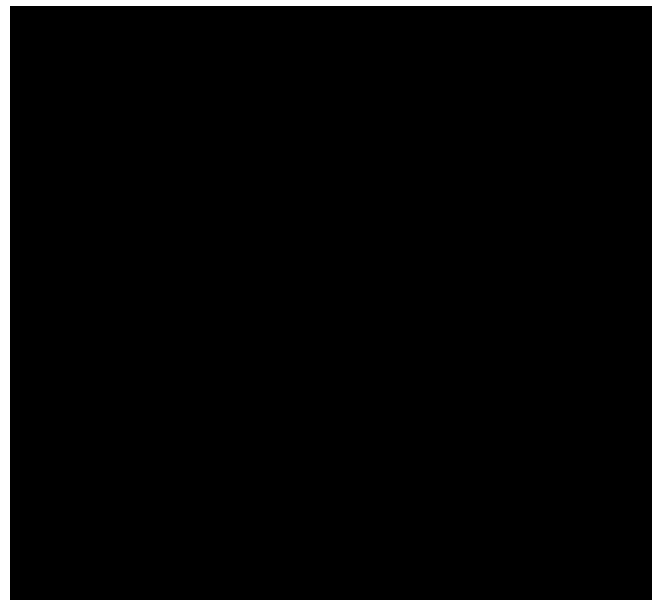


FIGURE 4. Dot plot showing the pAgNOR values of cases of ductal carcinoma in situ with (+ve) and without (-ve) invasion.

reproducible and requires no complicated procedures. Furthermore, the subclassification of comedo DCIS into low and high grades, based on nuclear morphology, has suggested the presence of a more aggressive subtype of DCIS. This method, however, did not show any significant impact on identifying DCIS lesions with more potential for invasive carcinoma.

Proliferative activity analysis of breast carcinoma is one of the most reliable independent prognostic indicators identifying more aggressive cases of breast carcinoma. This is especially seen in cases of breast carcinoma with diploid DNA indices.³³⁻³⁵ Methods of proliferative activity analysis include flow cytometry, immunohistochemical

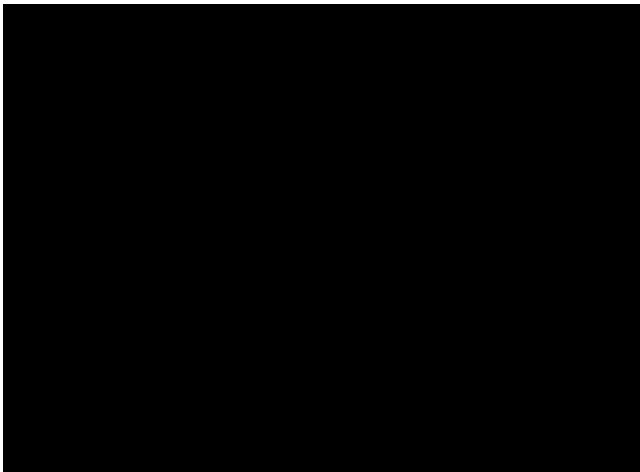


FIGURE 5. A case of ductal carcinoma in situ associated with invasion showing a high number of granule count (AgNOR silver stain, 40x).

staining for PCNA, Ki-67³⁶ and BrDU,³⁷ and the AgNOR silver stain.¹² We have previously shown that the AgNOR silver stain, together with the use of two counts for ploidy and proliferative activity analysis, correlate well with ploidy and proliferative activity measured by flow cytometry.²² We have also shown that, in DCIS, pAgNOR as a reflection of proliferative activity is the most significant predictor of invasion in DCIS.³⁸ This predictive value held in the current study on a different group of patients. Unfortunately, the correlation was not 100% predictive of invasive potential.

The pathogenesis of breast carcinoma is a multifactorial process.³⁹ This multifactorial background suggests that several factors play a role in determining the biological behavior of these neoplasms. This would also suggest that the development of invasive carcinoma in the background of DCIS is multifactorial as well. Our study suggests that when several biological factors are taken into account in combination rather than separately, a better idea could be obtained on the potential of invasive carcinoma occurring in DCIS. We used a very limited number of prognostic indicators in our study to attempt to predict the invasive potential of DCIS. We believe that the use of a larger number of well-established prognostic indicators in DCIS, such as other oncogenes,⁴⁰ estrogen and progesterone receptors,⁴¹ adhesion molecules⁴² and well-established histological criteria, would facilitate identification of DCIS lesions with high potential for invasive carcinoma. These high-risk lesions would probably require more aggressive management than ones with less potential for association with invasive cancer.

References

1. Rosner D, Bedwani RN, Vano J, Baoker HW, Murphy GP. Non-invasive breast carcinoma: results of a national survey by the American College of Surgeons. *Ann Surg* 1988;192:139-47.
2. Stacey-Clear A, McCarthy KA, Hall DA, Pile-Spellman E, White G, Hulka C, et al. Breast cancer survival among women under age 50: is mammography detrimental? *Lancet* 1992;340:991-4.
3. Carter D, Smith RRL. Carcinoma in situ of the breast. *Cancer* 1977;40:1189-93.
4. Lagios MD, Nestdahl PR, Margolin FR, Rose MR. Relationship of extent of non-invasive disease to the frequency of occult invasion, multicentricity, lymph node metastases, and short-term treatment failures. *Cancer* 1982;50:1309-14.
5. Page DL, Dupont WD, Rogers LW, Landenberger M. Intraductal carcinoma of the breast. Follow up after biopsy only. *Cancer* 1982;49:751-8.
6. Betsill WL, Rosen PP, Robbins GF. Intraductal carcinoma. Long-term follow up after treatment by biopsy only. *JAMA* 1978;239:1863-7.
7. Phipps RF, Rayter Z. In situ carcinoma of the breast. *Br J Hosp Med* 1990;44:168-70.
8. Schnitt SJ, Silen W, Sadowsky NL, Connolly JL, Harris JR. Ductal carcinoma in situ (intraductal carcinoma) of the breast. *N Engl J Med* 1988;318:898-903.
9. Rosen PP, Senie, Schottenfeld D, Ashikari R. Non-invasive breast carcinoma. *Ann Surg* 1979;189:377-82.
10. Rosen PP. Axillary lymph node metastases in patients with occult non-invasive breast carcinoma. *Cancer* 1980; 46:1298-1306.
11. Hardman PDJ, Worth A, Lee U. The risk of occult invasive breast cancer after excision biopsy showing in situ ductal carcinoma of comedo pattern. *Can J Surg* 1989;32:56-60.
12. Mourad WA, Sneige N, Singletary E, Sahin A, El Nagggar AK. The prognostic significance of two AgNOR counts in node-negative breast carcinoma (abstract). *Mod Pathol* 1992;5:16A.
13. Maguire HC, Hellman ME, Greene MI, Yeh MI. Expression of c-erbB-2 in in situ and adjacent ductal adenocarcinoma of the female breast. *Pathobiol* 1992;60:117-21.
14. Gasparini G, Bevilacqua P, Pozza F, Melis S, Boracchi P, Marubini E, Sainsbury JR. Value of receptor status compared with growth fraction and other factors for prognosis in early breast cancer. *Br J Cancer* 1992;66:970-6.
15. Rochefort H. Biological and clinical significance of cathepsin-D in breast cancer. *Semin Cancer Biol* 1990;1:153-160.
16. van Dongen JA, Holland R, Peterse JL, Fentiman IS, Lagios MD, Millis RR, Recht A. Ductal carcinoma in situ of the breast; second EORTC consensus meeting. *Eur J Cancer* 1992;28:626-9.
17. Lagios MD. Heterogeneity of ductal carcinoma in situ of the breast. *J Cell Biochem* 1993;17G:49-52.
18. Page DL, Lagios MD. Pathology and clinical evolution of ductal carcinoma in situ (DCIS) of the breast. *Cancer Let* 1994;86:1-4.
19. Holland R, Peterse JL, Millis RR, Eusebi V, Faverly D, van de Vijver MJ, et al. Ductal carcinoma in situ: a proposal for a new classification. *Sem Diagn Pathol* 1994;11:167-80.
20. Ploton D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet JJ. Improvement in the staining and the visualization of the argyrophilic proteins of the nucleolar organizer region at the optical level. *Histochem J* 1986;18:5-14.
21. Crocker J, Ayers J, McGovern J. Nucleolar organizer regions in small cell carcinoma of the bronchus. *Thorax* 1987;42:972-5.
22. Mourad WA, Erkman-Balis B, Livingston S, Shoukri M, Cox CE, Nicosia SV, Rowlands DT. Argyrophilic nucleolar organizer region in breast carcinoma. Correlation with DNA flow cytometry, histopathology, and lymph node status. *Cancer* 1992;69:1739-44.
23. Mourad WA, Katz RL, Sembera DL, Atkinson EN, El-Nagggar AK. Two AgNOR counts in fine needle aspirates of lymphoproliferative disorders compared with acridine orange flow cytometry. *Diagn Cytopathol* 1992;8:128-34.
24. Mourad WA, Connelly JC, Sembera DL, Atkinson EN, Bruner JM. The correlation of two AgNOR counting methods with bromodeoxyuridine

- labeling index. A study in metastatic tumors to the brain. *Hum Pathol* 1993;24:206-10.
25. Mourad WA, Sneige N, Ordonez NG, Katz RL. The correlation of two AgNOR counts with Ki-67 labeling index. A study in fine needle aspiration of breast carcinoma and lymphoproliferative disorders. *Diagn Cytopathol* 1994;10:113-9.
 26. Suresh UR, Cahwner L, Buckley CH, Fox H. Do AgNOR counts reflect cellular ploidy or cellular proliferation? A study of trophoblastic tissue. *J Pathol* 1990;160:213-5
 27. Menden Hall W, Schaffer R, Wackerly D. *Mathematical statistics with application*. 3rd ed. Boston: Duxbury Press, 1986:629-31
 28. Tandon AK, Clark GM, Chamness GC, Chirgwin TM, et al. Cathepsin D and prognosis in breast cancer. *N Engl J Med* 1990;65:265-71.
 29. Henry JA, McCarthy AL, Angus B, Westley BR, May FE, Nicholson S, et al. Prognostic significance of cathepsin D in breast cancer: an immunohistochemical study. *Cancer* 1990;65:265-71.
 30. Kraus MH, Fedi P, Starks V, Muraro R, Aaronson SA. Demonstration of ligand-dependent signaling by the erbB-3 tyrosine kinase and its constitutive activation in human breast tumor cells. *Proc Natl Acad Sci USA* 1993;90:2900-4.
 31. Allred DC, Clark GM, Tandon AK, Molina R, Tormey DC, Osborne CK, et al. HER-2/*neu* in node-negative breast cancer: prognostic significance of overexpression influenced by the presence of in situ carcinoma. *J Clin Oncol* 1992;10:599-605.
 32. Jarvinen TA, Kononen J, Peltto-Huikko M, Isola J. Expression of topoisomerase II alpha is associated with rapid cell proliferation, aneuploidy, and c-erbB-2 overexpression in breast cancer. *Am J Pathol* 1996;148:2073-82.
 33. Clark GM, Dressler LG, Owens MA, Pounds G, Oldaker T, McGuire WL. Prediction of relapse or survival in node-negative breast cancer. *N Engl J Med* 1990;322:1045-53.
 34. Merkel DE, McGuire WL. Ploidy, proliferative activity and prognosis. DNA flow cytometry of solid tumors. *Cancer* 1990;65:1194-205.
 35. McGuire DL. DNA flow cytometry and other prognostic factors in breast carcinoma. *Cancer Invest* 1990;8:245-6.
 36. Keshgegian AA, Cnaan A. Proliferation markers in breast carcinoma: mitotic figure count, S-phase fraction, proliferating cell nuclear antigen, Ki-67 and MIB-1. *Am J Clin Pathol* 1995;104:42-9.
 37. Christov K, Chew KL, Ljung BM, Waldman FM, Goodson WH, Smith HS, Mayall BH. Cell proliferation in hyperplastic and in situ carcinoma lesions of the breast estimated by in vivo labeling with bromodeoxyuridine. *J Cell Biol* 1994;19:165-72.
 38. Mourad WA, Setrakian S, Hales ML, Abdulla M, Trucco G. The argyrophilic nucleolar organizer regions in ductal carcinoma in situ of the breast. The significance of ploidy and proliferative activity analysis using this silver staining technique. *Cancer* 1994;74:1739-45.
 39. Smith HS, Lu Y, Deng G, Martinez O, Krams S, Ljung BM, Thor A, Lagios M. Molecular aspects of early stages of breast cancer progression. *J Cell Biochem* 1993;17G:144-52.
 40. Sheikh MS, Shao Z, Hussain A, Fontana JA. The p53-binding protein MDM2 gene is differentially expressed in human breast carcinoma. *Cancer Res* 1993;53:3226-8.
 41. Wilbur DC, Barrows GH. Estrogen and progesterone receptors and c-erbB-2 oncoprotein analysis in pure in situ breast carcinoma. An immunohistochemical study. *Mod Pathol* 1993;6:114-20.
 42. Basbridge SA, Gillet CE, Sampson JA, Walsh FS, Millis RR. Epithelial (E-) and placental (P-) cadherin cell adhesion molecule expression in breast carcinoma. *J Pathol* 1993;169:245-50.